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Influenza virosomes supplemented with GPI-0100 adjuvant: a potent vaccine formulation for antigen dose sparing

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Abstract

Adjuvants can stimulate vaccine-induced immune responses and can contribute decisively to antigen dose sparing when vaccine antigen production is limited, as for example during a pandemic influenza outbreak. We earlier showed that GPI-0100, a semi-synthetic saponin derivative with amphiphilic structure, significantly stimulates the immunogenicity and protective efficacy of influenza subunit vaccine administered via a systemic route. Here, we evaluated the adjuvant effect of GPI-0100 on a virosomal influenza vaccine formulation. In contrast to influenza subunit vaccine adjuvanted with GPI-0100, virosomal vaccine supplemented with the same dose of GPI-0100 provided full protection of mice against infection at the extremely low antigen dose of 2 x 8 ng hemagglutinin. Overall, adjuvanted virosomes elicited higher antibody and T cell responses than did adjuvanted subunit vaccine. The enhanced immunogenicity of the GPI-0100-adjuvanted virosomes, particularly at low antigen doses, is possibly due to a physical association of the amphiphilic adjuvant with the virosomal membrane. These results show that a combination of GPI-0100 and a virosomal influenza vaccine formulation is highly immunogenic and allows the use of very low antigen doses without compromising the protective potential of the vaccine.

Key words: influenza vaccine, GPI-0100, subunit, virosome

1. Introduction

For more than 60 years vaccination has been the primary strategy in the prevention of influenza virus infection [1-3]. Due to their low local reactogenicity, vaccines consisting of purified viral proteins, such as split or subunit vaccines, are currently preferred over whole inactivated virus (WIV) vaccines. Purified protein vaccines are as immunogenic as WIV vaccine in primed individuals. However, in unprimed subjects the immunogenicity of protein vaccines is relatively weak [3-5]. Vaccine immunogenicity can be enhanced by using adjuvant(s) which can boost immune responses against a specific antigen [6,7]. In addition, the use of adjuvants may allow antigen dose-sparing vaccination strategies which become very important in situations like pandemics in which vaccine demand may far exceed the available vaccine production capacity.

Our earlier studies show that GPI-0100, a semi-synthetic derivative of saponin, is a promising candidate adjuvant for influenza subunit vaccine. In a mouse model, GPI-0100 enhanced the antibody responses to intramuscularly injected subunit vaccine to a higher level than currently licensed adjuvants such as alum, MF59 or AS03 [8,9]. In addition, GPI-0100 effectively stimulated influenza-specific cellular immunity, which is crucial for immune memory formation and thus long-term protection. Remarkably, adjuvantation of subunit vaccine with GPI-0100 allowed a 25-fold reduction in hemagglutinin (HA) dose (down to 0.04 μg) without compromising the immunogenicity and protective capacity of the vaccine.

Chemically, GPI-0100 is an amphiphilic molecule containing a triterpenoid backbone with sugar moieties and a fatty-acid side chain attached via the carboxyl group to one of the sugars [10,11]. The presence of this hydrophobic moiety gives the molecule an amphiphilic character and might enable physical association of GPI-0100 with membrane-containing antigen formulations thus further potentiating its immuno-stimulating properties.

Influenza virosomes are reconstituted viral membrane envelopes. Virosome production involves three steps: (1) solubilization of the virus membrane with a proper detergent, (2) removal of the virus core proteins and genetic material by ultracentrifugation and (3) reconstitution of the virus membrane by detergent extraction [12,13]. Influenza virosomes retain the structural and functional properties of the viral membrane envelope. Yet, the lack of viral genomes clears any concern for viral replication and infection. Functionally preserved virosomes can bind to cellular sialic acid receptors for hemagluttinin (HA), initiate receptor-mediated endocytosis and deliver encapsulated agents to the cytosol [12,14-16]. The virosomal HA is degraded in endosomes, resulting in MHC class II presentation and CD4+ T cell activation, which helps the development of CTL and B cell responses [12,16]. In addition, the repetitive arrangement of HA spikes on the virosome membrane can cross-link membrane-bound antibodies expressed on B cells and give strong activation signals to these cells [12,15,17]. Invivac® and InflexalV® are licensed virosomal influenza vaccines. Both vaccines have been shown to be well-tolerated and to induce immune responses as good as or even better than conventional inactivated influenza virus vaccines [18,19]. Importantly, unlike subunit vaccines, influenza virosomes provide a lipid membrane platform for physical association of amphiphilic adjuvants with the virosomal particle. This allows antigen-presenting cells to be activated by adjuvant and exposed to antigen at the same time. An earlier study in Balb/c mice showed that a single administration of influenza virosomes with a Toll-like receptor 2 ligand incorporated in the membrane resulted in a 150-fold enhancement of IgG responses when compared to plain virosomes [15].

Here we evaluated the adjuvant effect of GPI-0100 on a virosomal influenza vaccine formulation as compared to a subunit vaccine formulation. To this end, mice were immunized intramuscularly (IM) with non-adjuvanted or GPI-0100-adjuvanted subunit or virosomal vaccine and immune responses were evaluated and the mice were challenged with a lethal dose of homologous virus. Our data show that GPI-0100 enhanced the immunogenicity and protective capacity of influenza subunit and virosome vaccines to different extents. Overall, the adjuvanted virosomes elicited stronger humoral and cellular immunity than the adjuvanted subunit vaccine, especially at low antigen doses. Notably,

only the adjuvanted virosomal, but not the subunit, vaccine remained fully protective at an antigen dose of 8 ng HA. We conclude that influenza virosomes admixed with GPI-0100 provide a simple and yet potent influenza vaccine formulation especially with regard to antigen dose sparing.

2. Materials and Methods

2.1 GPI-0100

GPI-0100 was purchased from Hawaii Biotech, Inc. (Aiea, HI, USA) as powder and was stored at 4 °C. The preparation and storage of GPI-0100 stock solution was described earlier [8].

2.2 Influenza virus and vaccines

A stock of A/Puerto Rico/8/34 (H1N1) influenza virus (PR8), propagated on Madin–Darby canine kidney (MDCK) cells, was kindly provided by Solvay Biologicals (currently Abbott Biologicals, Weesp, Netherlands). The virus was further propagated on embryonated chicken eggs, purified by sucrose gradient centrifugation, and the virus titer was determined by measuring the tissue-culture infectious dose 50 ($TCID_{50}$) as previously described [8].

Influenza virus was inactivated by β-propiolactone treatment followed by dialysis against HBS buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA, pH 7.4) as described earlier [8]. The inactivated virus sample was processed to subunit vaccine using treatment with Tween 80 (0.6 mg/ml) and cetyltrimethylammonium bromide (CTAB, 3.0 mg/ml) followed by ultracentrifugation and detergent removal [8].

Virosomes were prepared from the inactivated influenza virus with 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC), as described previously [20]. Briefly, inactivated PR8 virus (1.5 μmol of viral phospholipid) was solubilized in 750 μl of 200 mM DCPC (Avanti Polar Lipids, Inc, Alabaster, AL, USA) in HBS buffer. The suspension was incubated on ice for 30 min and the nucleocapsid was removed from the preparation by

ultracentrifugation. The DCPC was then removed by dialysis against HBS buffer overnight. The buffer was refreshed on the next day for another 4 hr of dialysis. Reconstituted virus membranes (virosomes) were separated from non-incorporated material on a discontinuous sucrose density gradient (10-50%, w/v) in HBS, in a SW55 rotor at 33,000 rpm for 90 min. Subsequently, virosomes were dialyzed against HBS buffer to remove sucrose. Sterility of vaccine preparations was verified by plating on blood/agar plates and incubating for 24 hr at 37°C.

The protein content of the subunit and the virosome vaccine was determined by a modified Lowry assay [21]. Hemagglutinin (HA) content was assumed to be equal to the total protein content since sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) proved that other proteins were present only in very minor amounts. Adjuvanted subunit or virosomal vaccines were prepared by mixing of the indicated amounts of antigen and GPI-0100 just before immunization.

2.3 Animal handling

The protocol for the animal experiments was approved by the Ethics Committee on Animal Research of the University of Groningen (Permit number: DEC 5896B and 5896C). Female Balb/c mice (Harlan, The Netherlands), aged 8–10 weeks, were grouped (n = 6 per group) and immunized IM with PR8 subunit or virosome vaccine alone or with GPI-0100 adjuvantation in a two-dose immunization regimen (day 0 and day 20). The amounts used are indicated in the Results section. Control mice were injected with HBS buffer. On day 27, mice were challenged by intranasal administration of 200 $TCID_{50}$ PR8 influenza virus in 50 μl of HBS buffer. Virus administration was carried out under isoflurane anesthesia to ensure deposition of the virus into the lungs. Mice were monitored twice a day at fixed time points for clinical signs of illness including weight loss, changes in behavior and appearance. Mice were bled and sacrificed on day 30. Serum samples were collected for ELISA assay. Spleens were harvested and splenocytes were used for ELISPOT assay. The lung lobes were collected in 1 ml PBS and the homogenized supernatants were stored at −80 °C freezer for lung virus titer detection.

2.4 ELISA

H1N1-specific antibody responses were determined by ELISA as described earlier [8]. Individual IgG titers were calculated as the ^{10}log of the reciprocal of the sample dilution corresponding to an OD492 of 0.2. Individual IgG1 and IgG2a responses are given as concentration (μg/ml).

2.5 Hemagglutination-inhibition (HAI) assay

Serum samples were processed and subjected to HAI assay as earlier described [20]. ^{2}log HAI titers for individual mice are presented.

2.6 Elispot assays

H1N1-specific IFN-γ and IL-4 responses were determined by Elispot assays as earlier described [8]. Numbers of influenza-specific IFNγ- or IL4-secreting cells per 500,000 splenocytes from individual mice are given.

2.7 Virus titration in lungs of challenged mice

Virus titers were determined from lung homogenates as earlier described [8]. Results from individual mice are presented as ^{10}log (virus titer per gram of lung tissue).

2.8 Statistics

The unpaired Student's t-test was used to determine if the differences in influenza-specific responses observed between groups of mice were significant. A p value of $p < 0.05$ was considered significant.

3. Results

3.1 Immunogenicity and protective capacity of influenza subunit or virosomal vaccine with or without GPI-0100

Preliminary evaluation of influenza vaccine in different formulations was conducted at an antigen dose of 200 ng HA. Mice were immunized IM. twice on day 0 and 20 with subunit or virosomal vaccine alone or in the presence of 30 μg GPI-0100. One week after

the second immunization, mice were challenged with homologous virus via the intranasal route. No significant weight loss was observed until sacrifice three days after challenge. Mock-immunized control mice developed an average lung virus titer of 10^6 $TCID_{50}$/g lung tissue (Figure 1A). The average titer was reduced by ≥ 4 logs in each of the immunized groups ($p \leq 0.0001$ for all comparisons of mock-immunized and immunized groups). None of the mice immunized with plain subunit vaccine developed sterile immunity, while in 67% of the mice immunized with GPI-0100-adjuvanted subunit vaccine lung virus titers were below the detection limit. For virosomal vaccine, complete lung protection against virus growth was observed in 50% and 100% of the mice receiving plain virosomes and GPI-0100-adjuvanted virosomes respectively.

We further analyzed HAI titers in serum samples collected from the mice described above. None of the mice that received plain influenza subunit vaccine developed a detectable serum HAI titer (Figure 1B). Only 1 out of the 6 mice receiving GPI-0100-adjuvanted subunit vaccine showed a detectable serum titer. On the other hand, influenza virosomes induced detectable HAI titers in 3 and 4 out of the 6 immunized mice for plain and GPI-0100-adjuvanted vaccine, respectively. The average HAI titer induced by the adjuvanted virosomes was significantly higher than that induced by adjuvanted subunit vaccine (p= 0.0304).

3.2 Protective capacity of GPI-0100-adjuvanted influenza subunit or virosomal vaccine at a reduced antigen dose

To gain better insight into possible differences between GPI-0100-adjuvanted subunit and virosomal vaccines, we repeated the challenge experiment using a lower dose of GPI-0100 (15 µg) and titrating the antigen dose down from 200 to 8 ng. No significant weight loss was observed three days after virus challenge. On day 4 post challenge, the non-immunized control mice all showed more than 10% of weight loss (Figure 2A). One out of the 6 mice immunized with adjuvanted low-dose (8 ng) subunit vaccine also demonstrated severe weight loss and two others lost ≥5% of weight. The rest of the immunized and challenged mice, however, showed no significant weight changes (except one animal in the group immunized with adjuvanted virosomes at 40 ng HA).

All mice were sacrificed four days after challenge and virus titers were determined in the collected lung homogenates. The control mice developed an average lung virus titer of $10^{7.2}$ $TCID_{50}$/g lung tissue (Figure 2B). All mice that received adjuvanted vaccines containing more than 40 ng HA were completely or nearly completely protected fromvirus growth in the lungs regardless of the vaccine formulation used. At the lowest tested antigen dose of 8 ng HA, a significant difference in lung protection was observed between mice immunized with adjuvanted subunit or adjuvanted virosomal vaccine (p= 0.0078). Though the lung virus titer in subunit-immunized mice was significantly reduced as compared to that in the control group (p=0.0096), only 2 out of the 6 immunized mice developed sterile immunity with lung virus titers below the detection limit. The other mice of the group, however, showed only partial or no protection. In contrast, GPI-0100-adjuvanted virosomes provided full protection in all vaccinated mice even at the lowest antigen dose.

3.3 Humoral immunogenicity of GPI-0100-adjuvanted subunit or virosomal vaccine at a reduced antigen dose

Humoral immune responses elicited by GPI-0100-adjuvanted subunit or virosomal vaccines at different antigen doses were evaluated by performing H1N1-specific IgG ELISAs and HAI assays on serum samples collected from the mice described above under 3.2. Pre-challenge serum samples collected on day 27 showed that GPI-0100-adjuvanted subunit and virosomal vaccine elicited comparable H1N1-specific IgG responses at an antigen dose of 200 ng HA (Figure 3A). However, at an antigen dose of 40 ng there was a trend towards higher IgG responses in the virosome group as compared to those in the subunit group. This trend became statistically significant at the lowest antigen dose of 8 ng (p = 0.0091).

HAI responses were measured in post-challenge serum samples collected upon sacrifice of the animals. Titers were clearly antigen dose-dependent (Figure 3B). All mice that received 200 ng HA + GPI-0100 developed high HAI titers. At an antigen dose of 40 ng HA, measurable HAI titers were detected in 3 out of 6 or 5 out of 6 mice receiving the

adjuvanted subunit or virosomal vaccine, respectively. For these two antigen doses, HAI titers elicited by the virosomal vaccine were significantly higher than those elicited by the subunit vaccine (p= 0.0128 and 0.0026, respectively). At 8 ng HA, HAI titers elicited by either of the vaccines were low. Only 1 of the subunit-immunized mice and 3 of the virosome-immunized mice developed detectable HAI titers. Thus, overall GPI-0100-adjuvanted virosomes induced higher levels of influenza-specific antibodies than GPI-0100-adjuvanted subunit vaccine.

3.4 Phenotype of the antibody response elicited by GPI-0100-adjuvanted subunit and virosomal vaccine

We further analyzed the phenotype of the antibody response by performing H1N1-specific IgG1 and IgG2a ELISAs on the pre-challenge serum samples mentioned in 3.3. GPI-0100-adjuvanted subunit and virosomal vaccine elicited similar serum IgG1 responses at all antigen doses tested (Figure 4A). On the other hand, the vaccine formulation did play a role in the induction of serum IgG2a (Figure 4B). While at higher antigen doses, the adjuvanted subunit vaccine was equally effective as the virosomal vaccine in induction of IgG2a, only the adjuvanted virosomes readily induced IgG2a at the low antigen dose of 8 ng HA, resulting in a significant difference between these two groups (p=0.0289). Nevertheless, for both formulations a Th2-oriented antibody response predominated since high levels of IgG1 were elicited at any given antigen dose.

3.5 Cellular immunity of GPI-0100-adjuvanted subunit or virosomal vaccine at different antigen doses

We next evaluated H1N1-specific cellular immunity elicited by the GPI-0100-adjuvanted vaccines by performing Elispot assays on post-challenge splenocytes collected upon sacrifice. H1N1-specific IFN-γ responses elicited by the adjuvanted subunit vaccines were generally very weak or undetectable (Figure 5A). The adjuvanted virosomes, on the other hand, effectively induced H1N1-specific IFN-γ responses at an antigen dose of 8 and 40 ng HA, with an average of 69 and 136 IFN-γ-secreting cells per $5x10^5$ splenocytes, respectively. Unexpectedly, H1N1-specific IFN-γ-secreting cells were barely induced in the adjuvanted 200 ng HA virosome group. In the animal groups that received 40 ng HA,

a trend towards higher IFN-γ responses for the adjuvanted virosomes as compared to the subunit vaccine was observed. This trend reached statistical significance at 8 ng HA (p= 0.0016). All of the vaccines tested readily induced H1N1-specific IL-4 responses after challenge. A strong antigen dose-dependent IL-4 response was observed for GPI-0100-adjuvanted subunit vaccine (Figure 5B). At a high antigen dose of 200 ng HA, the adjuvanted subunit vaccine elicited significantly stronger IL-4 responses than did the adjuvanted virosomes (p=0.0023). At lower antigen doses, however, the adjuvanted virosomes elicited significantly stronger IL-4 responses than the adjuvanted subunit vaccine (p=0.0436 and 0.0094 for a comparison at 8 and 40 ng HA, respectively). Notably, Th1-skewed cellular immunity, predominated by IFN-γ-producing T cells, was observed only in mice that received GPI-0100-adjuvanted virosomes at an antigen dose of 40 ng HA or lower. Thus, the adjuvanted virosomes elicited a stronger and more Th1-oriented cellular immune response than did the adjuvanted subunits at a low antigen dose.

4. **Discussion**

In an attempt to further improve the immunogenicity of influenza vaccines we compared in this study the effects of GPI-0100 adjuvantion on a subunit and a virosomal influenza vaccine formulation. Due to its amphiphilic nature GPI-0100 can potentially incorporate into virosomal membranes thus forming particles containing both antigen and adjuvant. In the absence of GPI-0100 subunit and virosomal vaccines induced similar levels of antibodies and reduction in lung virus titer after challenge. GPI-0100 stimulated the protective efficacy of both formulations but to different extents. At limiting amounts of antigen (8 ng HA) only the adjuvanted virosomal vaccine could completely prevent infection. Furthermore, the adjuvanted virosomes induced higher antibody titers and higher numbers of cytokine-producing T cells than adjuvanted subunit vaccine at antigen doses of 40 ng or lower,. These differences were not detected when an antigen dose of 200 ng was used, presumably because at this relatively high dose of antigen, the nature of the vaccine formulation becomes less critical. Thus, overall GPI-0100-adjuvanted virosomes were superior to adjuvanted subunit vaccine, particularly at low antigen doses.

Exploitation of the amphiphilic nature of saponin adjuvants to develop immunogenic vaccine delivery systems has been reported earlier. Immune-stimulating complexes (ISCOMs), composed of saponin, phospholipid, cholesterol and incorporated antigen are among the most potent saponin-containing formulations identified [24,25]. The ISCOM formulation contains cage-like particles approximately 40 nm in diameter. A challenge study in mice showed that subcutaneous delivery of a single dose of ISCOMs consisting of the saponin ISCOPREP^{TM}703 and PR8 antigen protects mice from weight loss and death at an antigen dose of 0.5 µg HA [26]. Another study in mice using ISCOMs containing the saponin Quil A and the antigen from A/Taiwan/1/86 (H1N1) virus demonstrated that two subcutaneous immunizations with an antigen dose of 0.5 µg HA were required to provide complete lung protection against homologous challenge [27]. Here we show that mice receiving two intramuscular immunizations of GPI-0100-adjuvanted PR8 virosomes were protected from weight loss and lung infection upon challenge even at the very low antigen dose of 8 ng HA. Hence, GPI-0100-adjuvanted virosomes provide a formulation which is relatively simple, exploits a marketed influenza vaccine formulation, is easy to produce, and at the same time performs as good as or even better than influenza ISCOMs.

Influenza virosomes are 100-200 nm particles composed of the membrane lipids and spike proteins of influenza virus. Unlike influenza virus-like particles (VLPs), which are produced by infecting cells lines with genetically modified viral vectors containing influenza virus genes, influenza virosomes are produced from cultured influenza viruses with or without addition of extra lipids [13,17,28,29]. The safety and efficacy of virosomal influenza vaccines has been demonstrated to be as good as or even better than that of split or subunit vaccines in children, adults and healthy elderly or those with a medical condition [19,30-34]. Importantly, due to their membranous nature, virosomal vaccines provide a platform for the incorporation of lipophilic or amphiphilic adjuvants [35-40]. Such an integration of stimulatory signals from both antigen and adjuvant is expected to elicit robust immune responses [41,42].

Adjuvanted virosomal vaccines can be produced by different methods. One way is to combine lipophilic/amphiphilic adjuvant and virosomal antigen during the process of virosome reconstitution. The adjuvant is mixed with solubilized viral membranes in the presence of solubilization agent. After incubation, the mixture is dialyzed against buffer for the removal of solubilization agent and formation of virosomes. Using this method, respiratory syncytial virus (RSV) virosomes with incorporated P3CSK4 or MPLA (TLR-2 and TLR-4 ligand respectively) were prepared. The adjuvant-modified RSV virosomes were shown to induce significantly stronger Th1 immunity than non-adjuvanted virosomes and formalin-inactivated RSV and to induce full protection against RSV infection [35,36]. Addition of an amphiphilic adjuvant prior to virosome reconstitution has also been used successfully for the generation of influenza vaccines. A study on virosomal H5N1 vaccine shows that incorporation of the LPS-derivative LpxL1 stimulates vaccine immunogenicity and skews immune responses towards a Th1 phenotype [37]. Alternatively, adjuvants can be simply added to preformed virosomal vaccines. Cox et al. showed that addition of ISCOMATRIX (Matrix-M^{TM}) to preformed H5N1 virosomes prior to injection significantly enhances the immunogenicity of the vaccine in both mice and humans [38-40]. Murine studies further show that Matrix-M-adjuvanted virosomes elicit much stronger Th1 responses (IgG2a, IL-2, IFN-γ and IL-12) and higher frequencies of multifunctional Th1 CD4+ cells when compared to plain virosomes. In addition, for intranasal delivery only the Matrix-M-adjuvanted but not plain virosomes provide protection against homologous virus infection. The effectiveness of a simple admixture of adjuvant and virosomal antigen is further clarified in our study. Addition of GPI-0100 to preformed virosomal H1N1 vaccine prior to immunization was sufficient to strongly potentiate immune responses.

While GPI-0100 does enhance immune responses when combined with influenza subunit vaccine which largely lacks lipids, our results indicate that it is considerably more potent when admixed with virosomes which consist of reconstituted viral membranes. We hypothesize that the potent immunogenicity and antigen dose-sparing capacity of GPI-0100-adjuvanted virosomes are due to a physical association of the amphiphilic adjuvant molecule with the virosomal membrane. Yet, we so far do not have formal proof for this

hypothesis. Preliminary results from particle size determination by use of Nanosight equipment show that GPI-0100 changes the average particle size of influenza virosomal but not subunit vaccines. Whether the observed phenomenon is caused by GPI-0100 partitioning into the virosomal membrane vesicles or by reformation of GPI-0100-disrupted virosomes, or by a combination of both, needs to be elucidated.

In summary, we show that GPI-0100 is a very potent adjuvant when used in combination with virosomal influenza vaccine. Particularly at limiting amounts of antigen, GPI-0100 adjuvanted virosomes elicited higher antibody titers and higher numbers of IFNγ-producing T cells than equal amounts of adjuvanted subunit vaccine. Remarkably, complete lung protection against homologous challenge was achieved by two immunizations with only 8 ng HA formulated in adjuvanted virosomes. This indicates that adjuvantation of virosomal influenza vaccine with GPI-0100 is a very promising strategy for antigen dose sparing as required in case of influenza pandemics. Yet, further characterization of the vaccine formulation and optimization of the ratio between GPI-0100 and the virosomal phospholipids are required before clinical application can be envisaged.

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7. Figure legends

Figure 1. Lung protection and hemaglutination inhibition (HAI) titer elicited by influenza subunit or virosomal vaccine with and without GPI-0100.

Mice (n=6 per group) were immunized intramuscularly on day 0 and day 20 with 0.2 μg A/PR/8 subunit or virosomal vaccine alone or adjuvanted with 30 μg GPI-0100. The control mice received HBS buffer. Mice were infected with live PR8 virus one week after the second immunization and were sacrificed 3 days after challenge for *ex vivo* analysis. (A) Lung virus titers. Virus titers are expressed as the ^{10}log virus titer per gram of lung tissue for individual mice. The black line represents the geometric mean virus titer per group. Due to technical reasons, only 4 or 5 samples from mice receiving plain or GPI-0100-adjuvanted virosomes, respectively, were available for lung virus titration. (B) Post-challenge HAI titers. The results are expressed as the ^{2}log HAI titers for individual mice. The black line represents the geometric mean HAI titer per group. The dotted line represents the detection limit. The stars indicate statistical differences between compared groups. Levels of significance are depicted as follows: *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.005$.

Figure 2. Protective capacity of GPI-0100-adjuvanted influenza subunit or virosomal vaccine at different antigen doses.

Mice were immunized intramuscularly on day 0 and day 20 with PR8 subunit or virosomal vaccine at different antigen doses (8, 40, 200 ng HA), adjuvanted with 15 μg GPI-0100. Control mice received HBS buffer. Mice were infected with live PR8 virus one week after the second immunization and were sacrificed 3 days after challenge for *ex vivo* analysis. (A) Weight changes. The weight upon sacrifice (day 30) was compared to the weight prior to challenge (day 27) for each individual mouse. The black line represents the arithmetic mean of the relative weight change per group. (B) Lung virus titer. Determined as described in the legend to Figure 1.

Figure 3. Humoral immune responses to GPI-0100-adjuvanted influenza subunit or virosomal vaccine at different antigen doses.

Pre- and post-challenge serum samples from the mice described in the legend to Fig. 2 were collected on day 27 and 37, respectively. A) Total IgG responses after two immunizations. ^{10}log IgG titers of individual mice are given. The black line represents the geometric mean IgG titer per group. (B) Post-challenge HAI titers. Due to technical reasons, only 4 samples from the mice that received 8 ng HA subunit adjuvanted with 15 μg GPI-0100 were available for the HAI assay.

Figure 4. Phenotype of the influenza-specific antibody responses to GPI-0100-adjuvanted influenza subunit or virosomal vaccine.

Serum samples from the mice described in the legend to Fig. 3(A) were analyzed. (A) Influenza-specific IgG1 (μg/ml) of individual mice with arithmetic mean per group. (B) Influenza-specific IgG2a (μg/ml) of individual mice with arithmetic mean per group.

Figure 5. Cellular immune responses to GPI-0100-adjuvanted influenza subunit or virosomal vaccine at different antigen doses.

Spleen samples from the mice described in the legend to Fig. 2 were collected on day 37. Splenocytes were isolated and stimulated overnight with PR8 subunit antigen. (A) IFN-γ-producing splenocytes per $5x10^5$ cells of individual mice with arithmetic mean per group. (B) IL-4-producing splenocytes per $5x10^5$ cells of individual mice with arithmetic mean per group.

Figure 1

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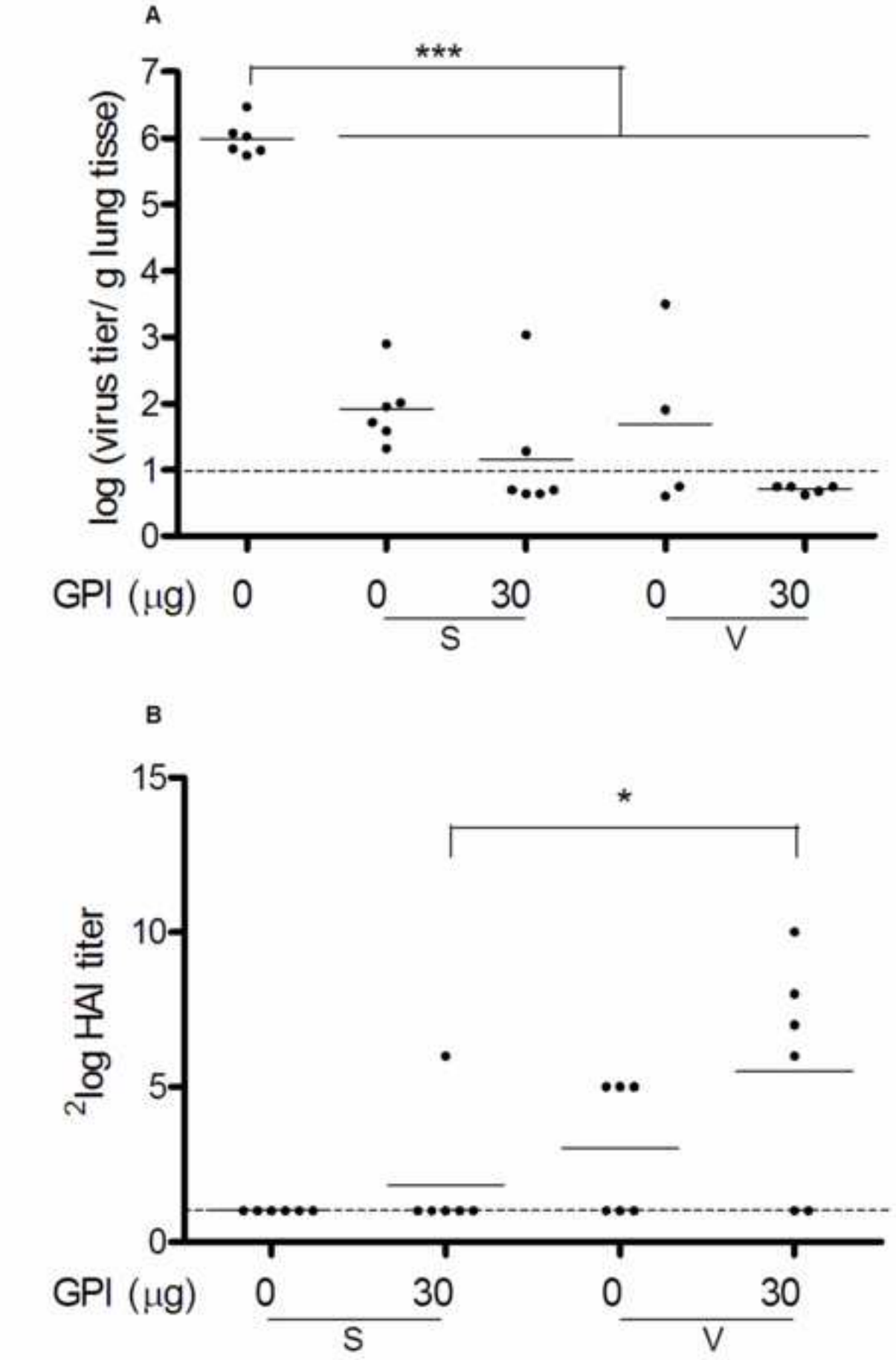

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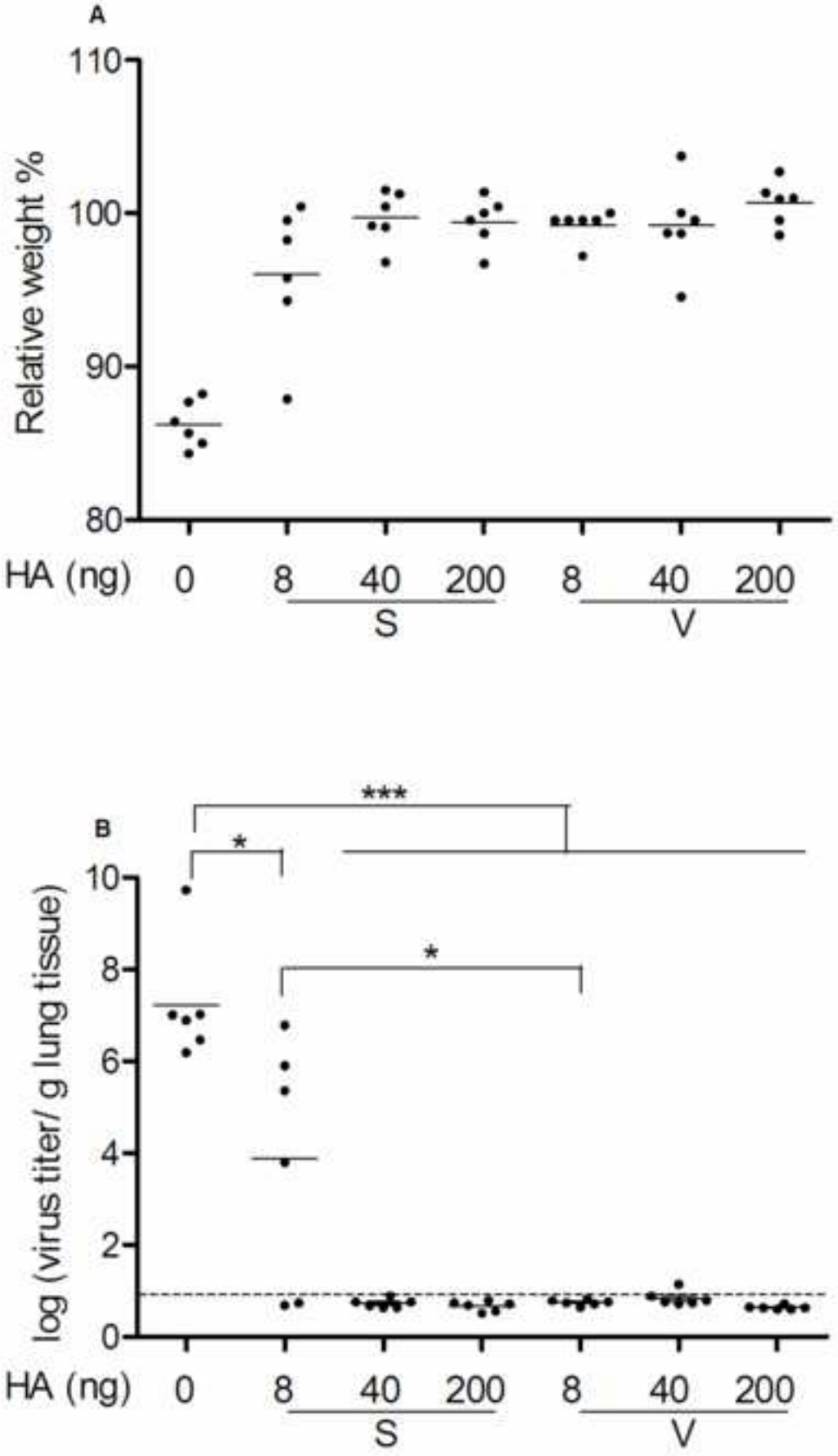

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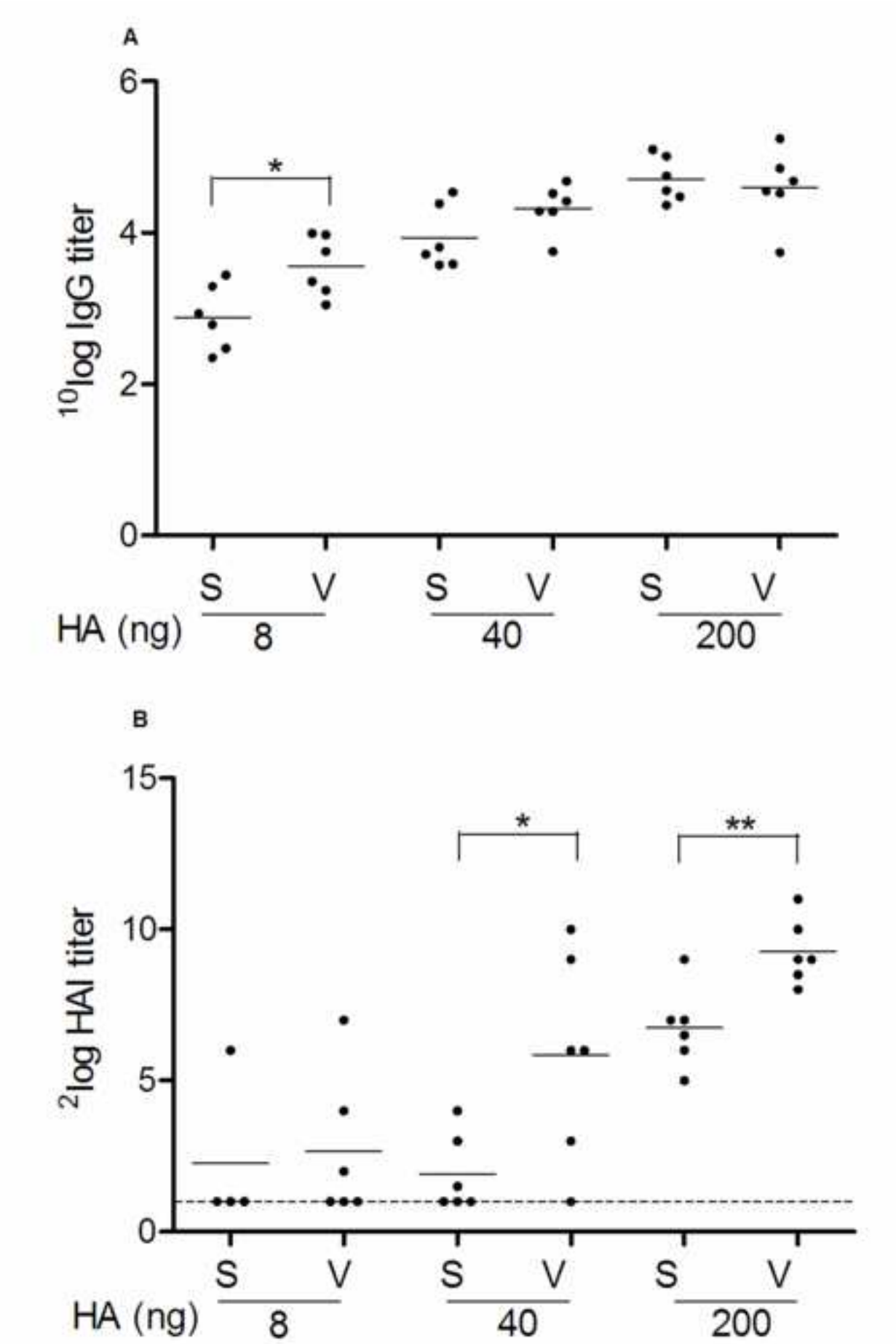

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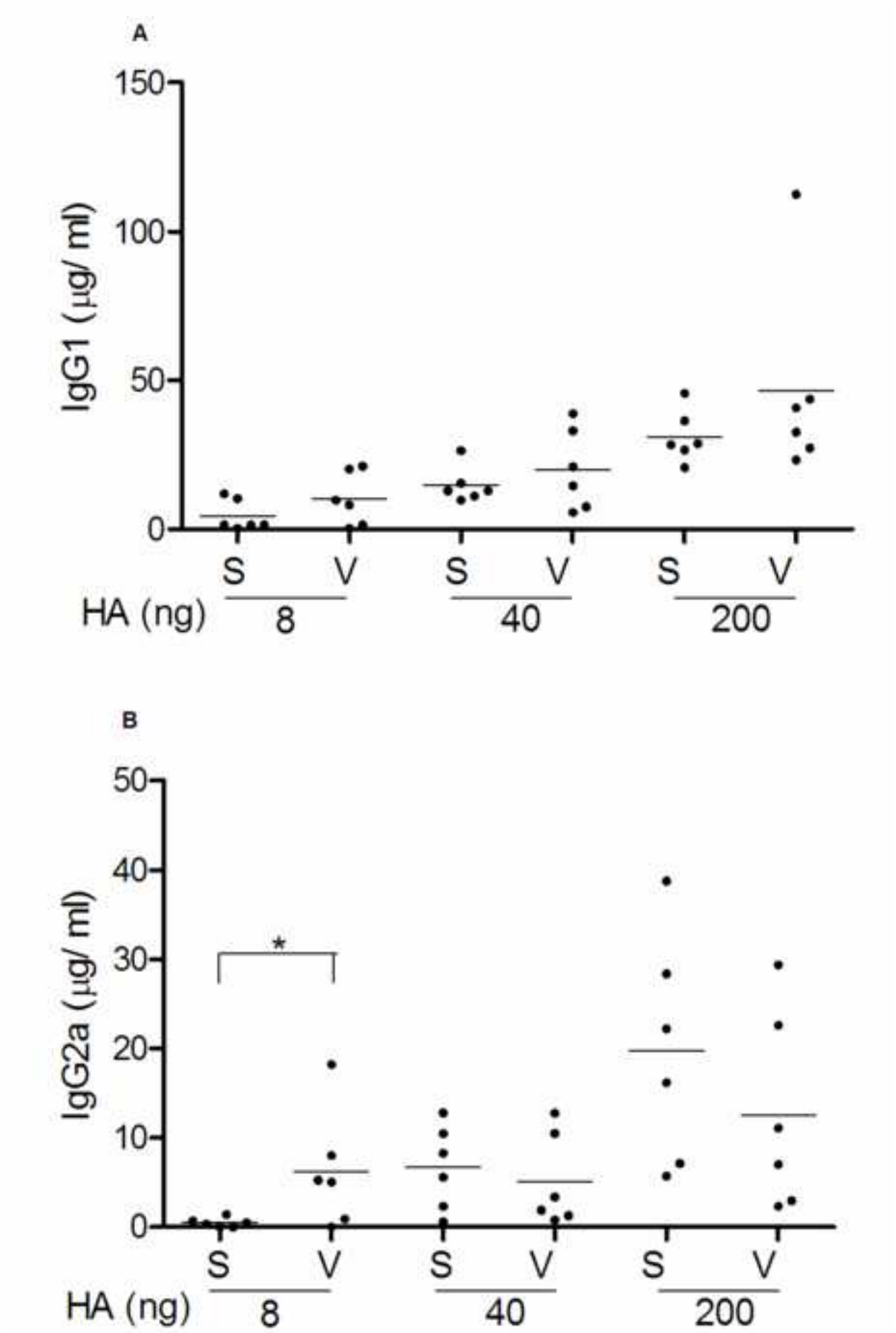

Figure 5
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